REPORT

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

WITH

[redacted]

(CONTACT TIME: 30 MINUTES)

NOTOX Project 338794
NOTOX Substance 111834

CONFIDENTIALITY STATEMENT

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STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director

Ing. M.J.E. Desmares-Koopmans

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Date: January 21.2002.

Management

Ing. E.J. van de Waart M.Sc.
Head of Genetic &
Ecotoxicology

.....

Date: 22/01/2002

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below.
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS AUDITS	REPORTING DATES
on-site inspection (s)	
15 October to 17 October 2001 (process)	25 October 2001
protocol inspection (s)	
13 November 2001 (study)	13 November 2001
report audit (s)	
17 January 2002 (study)	17 January 2002

Head of Quality Assurance C.J. Mitchell B.Sc.

Date: 23-Jan-02

SUMMARY

The influence of [redacted] on the respiration rate of activated sludge was investigated after a contact time of 30 minutes.

The study procedure was based on OECD Guideline No. 209, adopted April 4, 1984 and EEC Directive 67/548 amended November 18, 1987 (87/302), Part C, Publication No. L133, adopted May 30, 1988.

[redacted] s a clear colourless liquid and was treated as 100% pure.
The test substance was hardly soluble in water and was added quantitatively to the test vessels. A concentration range of 32 to 320 mg/l forming a geometric progression with a factor 1.8 was tested. 100 mg/l was tested in duplicate.

The inhibitory effect of [redacted] on aerobic waste water (activated sludge) bacteria increased with increasing concentration, ranging from 27% inhibition at the lowest concentration tested (32 mg/l) to 92% inhibition at the highest concentration tested (320 mg/l).

The EC-values were determined using linear regression (regression line: Y = 67.38 X –71.91, Y = % inhibition and X = log concentration (mg/l)).

The respiration rates of the controls were within 15% of each other.
The EC50 of the reference substance, 3,5-dichlorophenol, was 11 mg/l.
Therefore, the test was considered to be valid.

In conclusion, under the conditions of this present test, [redacted] was toxic to waste water (activated sludge) bacteria at and above 32 mg/l, the lowest concentration tested.

The EC10 was 16 mg/l with a 95% confidence interval ranging from 9 to 31 mg/l.
The EC20 was 23 mg/l with a 95% confidence interval ranging from 13 to 41 mg/l.
The EC50 was 65 mg/l with a 95% confidence interval ranging from 40 to 103 mg/l.
The EC80 was 180 mg/l with a 95% confidence interval ranging from 111 to 290 mg/l.

PREFACE

Sponsor	[redacted]
Study Monitor	Dr. C.L.J. Braun SHERA, Regulatory Affairs
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.J.E. Desmares-Koopmans
Technical Coordinator	J.H.J.W. Kluytmans
Study Plan	Start and completion: December 07, 2001

TEST SUBSTANCE

Identification Chemical name	[redacted]
CAS RN	Not yet assigned
Description	Clear colourless liquid
Batch	VRS01048
Purity	Treat as 100% pure
Test substance storage	In refrigerator in the dark
Stability under storage conditions	Not indicated
Expiry date	12 July 2002 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	1.1539 (determined at NOTOX)
Stability in water	Not indicated

The sponsor is responsible for all test substance data unless determined by NOTOX.

PURPOSE

The principal objective of this test method is the assessment of the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of test substance.
The test method is a rapid screening method whereby test substances which may adversely affect aerobic microbial treatment plants can be identified.

GUIDELINES

The study procedures described in this report were based on the following guidelines:

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 2: Effects on biotic systems, Guideline no. 209, "Activated Sludge, Respiration Inhibition Test", adopted April 4, 1984.

European Economic Community (EEC), EEC Directive 67/548 amended November 18, 1987 (87/302), Part C: Methods for the determination of ecotoxicity, Publication No. L133, "Biodegradation: Activated sludge respiration inhibition test", adopted May 30, 1988.

ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test substance reference sample and raw data.
No data will be withdrawn without the sponsor's written consent.

DEFINITIONS

Activated sludge	:	the accumulated biological mass produced in the treatment of waste water by the growth of bacteria and other micro-organisms in the presence of dissolved oxygen.
Suspended solids	:	the solids removed from activated sludge by filtration and dried to a constant mass, expressed in grams per litre.
Respiration rate	:	the oxygen consumption of aerobic activated sludge or waste water micro-organisms expressed generally as mg O_2 per litre per hour.
EC50	:	the concentration of the test substance at which the respiration rate is 50% of the respiration rate of the controls under the conditions of the test.

TEST SYSTEM

Test System	Micro-organisms in activated sludge.
Source	Municipal sewage treatment plant: 'Waterschap de Maaskant', 's-Hertogenbosch, the Netherlands.
Number of micro-organisms	Number of micro-organisms was determined as the amount of Mixed Liquor Suspended Solids (MLSS) per litre test medium.
Preparation of the sludge	The sludge was coarsely sieved, washed and diluted with tap-water. A small amount of the sludge was weighed and dried at ca. 105°C to determine the amount of suspended solids (3.2 g/l of sludge, as used for the test). Before use the pH was checked (measured value: 7.5). The batch of sludge was used on subsequent days (maximum four days), therefore 50 ml of synthetic sewage feed was added to each litre of activated sludge at the end of each working day. The sludge was kept aerated at test temperature until use.

Rationale	Recognized by international guidelines as the recommended test system.

TEST SUBSTANCE CONCENTRATIONS

[redacted]s a clear colourless liquid and was treated as 100% pure.
The test substance was hardly soluble in water and was added quantitatively to the test vessels.
A concentration range of 32 to 320 mg/l forming a geometric progression with a factor 1.8 was tested. 100 mg/l was tested in duplicate.
Concentrations were approved by the study director in the study files.

REFERENCE SUBSTANCE CONCENTRATIONS

A solution of 3,5-dichlorophenol (Aldrich, Cat. no. D 7,060-0, purity 97%) was prepared by dissolving 251.0 mg in 5 ml 1N NaOH, diluting it to approximately 15 ml with Milli-Q water and adding under stirring 1N H_2SO_4 to the point of incipient precipitation.
Finally the solution was diluted to 250 ml with Milli-Q water. The pH was 8.0.
Three concentrations were tested: 3.2, 10 and 32 mg/l.

TEST PROCEDURE AND CONDITIONS

Contact time	30 minutes, during which aeration and stirring took place.
Vessels	All glass, 300 ml oxygen bottles and 1 l test bottles.
Milli-RO / Milli-Q water	Tap-water purified by reverse osmosis (Milli-RO) and subsequently passed over activated carbon and ion-exchange cartridges (Milli-Q) (Millipore Corp., Bedford, Mass., USA).
Synthetic sewage feed	16 g peptone 11 g meat extract 3 g urea 0.7 g NaCl 0.4 g $CaCl_2.2H_2O$ 0.2 g $MgSO_4 .7H_2O$ 2.8 g K_2HPO_4 Dissolved in 1 l Milli-Q water and filtered. The pH was 7.0.
Air supply	Clean, oil-free air.
Oxygen meter	Oxygen electrode (Tri Ox EO 200, WTW, FRG) supplied with a recorder (Kipp BD40).
Performance of the test	The synthetic sewage feed (16 ml) and an adequate amount of the test substance were mixed and made up to 300 ml with Milli-RO water. Activated sludge (200 ml) was added and the mixture was aerated in a

1 l bottle during the contact time, using a pipette as an aeration device.

Then a well mixed sample of the contents was poured into a 300 ml oxygen bottle, and the flask was sealed with an oxygen electrode connected to a recorder, forcing the air out of the vessel. Oxygen consumption was measured and recorded for approximately 10 min. During measurement, the sample was not aerated but continuously stirred on a magnetic stirrer. The pH was determined in the remaining part of the reaction mixture.

This procedure was repeated for all concentrations of the test substance. In each test series two controls without test substance were tested, one at the start and one at the end.

Each batch of activated sludge was checked for sensitivity by testing the reference substance 3,5-dichlorophenol.

The flasks were filled up to 500 ml final volume, according to the scheme given below.

Flask	Synth. Sewage Feed (ml)	Stock sol. R (ml)	T (mg)	Made up to 300 ml with Milli-RO water	Activated sludge (ml)	Conc. (mg/l)
C1	16	-	-	+	200	-
R1	16	3.2	-	+	200	3.2
R2	16	10	-	+	200	10
R3	16	32	-	+	200	32
T1	16	-	16.0	+	200	32
T2	16	-	27.8	+	200	56
T3	16	-	50.2	+	200	100
T4	16	-	49.3	+	200	100
T5	16	-	90.6	+	200	180
T6	16	-	161.9	+	200	320
C2	16	-	-	+	200	-

C : Control.
R : Reference substance, 3,5-dichlorophenol.
T : Test substance, TRIGONOX R-938, quantitatively added.
Chronological order: T3 -> T4 -> T1 -> T2 -> T5 -> T6.

MEASUREMENT AND CALCULATIONS

The respiration rate from each vessel, in mg/l/hr, was calculated from the linear part of the respiration curve, which was in general between 2.5 and 6.5 mg O_2/l. The inhibitory effect (percentage inhibition) at a concentration was calculated as:

$$\% \text{ inhibition} = \left(1 - \frac{2 * Rt}{Rc \text{ (start test series)} + Rc \text{ (end test series)}}\right) * 100$$

in which Rc and Rt were respiration rates of controls and test/reference substance respectively (in mg/l/hr).

The percentage inhibition was plotted against the logarithm of the concentrations and the EC50, and if applicable the EC10, EC20 and EC80 for the test substance, were determined using linear regression analysis.

A figure of more than 10% inhibition was considered as significant.

ACCEPTABILITY OF THE TEST

The test was considered to be valid when:
- the respiration rates of both controls were within 15% of each other,
- the EC50 of 3,5-dichlorophenol was in the accepted range of 5-30 mg/l.

RESULTS

Toxicity o[redacted]

pH, oxygen concentration at the start of measurement and the influence o[redacted] on the oxygen consumption of microbes in activated sludge and percentage inhibition in respiration rate are presented in Tables 1, 2 and Figure 1.

The inhibitory effect of [redacted]n aerobic waste water (activated sludge) bacteria increased with increasing concentration, ranging from 27% inhibition at the lowest concentration tested (32 mg/l) to 92% inhibition at the highest concentration tested (320 mg/l).

The EC-values were determined using linear regression (regression line: Y = 67.38 X –71.91, Y = % inhibition and X = log concentration (mg/l)).

The EC10 was 16 mg/l with a 95% confidence interval ranging from 9 to 31 mg/l.
The EC20 was 23 mg/l with a 95% confidence interval ranging from 13 to 41 mg/l.
The EC50 was 65 mg/l with a 95% confidence interval ranging from 40 to 103 mg/l.
The EC80 was 180 mg/l with a 95% confidence interval ranging from 111 to 290 mg/l.

Experimental conditions

The temperature of the sludge, stored overnight in the laboratory, was 23.4°C at the start of the day of testing. Thus, this temperature was 1.4°C above the protocolled range.
Thereafter the sludge was transferred to a climate room. The study was performed in this climate room. The temperature in this room was 19.5°C.
The deviation at the start of the day of testing was considered to have no effect on the outcome of this study.

Acceptability of the test

The results are summarized in Tables 1, 3 and Figure 2.

The mean respiration rate of control 1 and 2 was 36 mg O_2/l/hr. The difference between the controls was 8%.

The EC50 of 3,5-dichlorophenol was 11 mg/l (regression line: Y = 63.95 X -16.84, Y = % inhibition and X = log concentration (mg/l)).

Since all criteria for acceptability of the test were met, this study was considered to be valid.

CONCLUSION

Under the conditions of this present test, [redacted] was toxic to waste water (activated sludge) bacteria at and above 32 mg/l, the lowest concentration tested.

The EC10 was 16 mg/l with a 95% confidence interval ranging from 9 to 31 mg/l.
The EC20 was 23 mg/l with a 95% confidence interval ranging from 13 to 41 mg/l.
The EC50 was 65 mg/l with a 95% confidence interval ranging from 40 to 103 mg/l.
The EC80 was 180 mg/l with a 95% confidence interval ranging from 111 to 290 mg/l.

Table 1: pH, oxygen concentration at the start of measurement and the influence of 3,5-dichlorophenol and [redacted] on the oxygen consumption of microbes in activated sludge and percentage inhibition in respiration rate.

Flask	Concentration reference/test substance (mg/l)	Oxygen conc. at the start (≡ mg O_2/l)	Oxygen consumption mg O_2/l/hr	Inhibition %	pH
C1	-	8.0	34	-	8.0
C2	-	6.5	37	-	8.1
Mean C1 + C2			36 (Δ8%)		
R1	3.2	8.5	31	13	8.0
R2	10	9.7	17	52	8.1
R3	32	9.5	8	77	8.1
T1	32	7.8	26	27	7.9
T2	56	7.7	20	44	7.9
T3	100	8.5	11	69	8.0
T4	100	8.5	13	63	8.0
T5	180	8.7	6	83	7.9
T6	320	9.4	3	92	8.0

C : Control.
R : Reference substance, 3,5-dichlorophenol.
T : Test substance [redacted]

Table 2: Determination of the EC50-value for [redacted]

Parameter: Inhibition of respiration

Conc (mg/l)	X Log conc. (mg/l)	Y Inhibition (%)
32	1.505	27
56	1.748	44
100	2.000	69
100	2.000	63
180	2.255	83
320	2.505	92

Regression parameters

Slope:	67.3786
Intercept:	-71.9117
Multiple R:	0.9862
n = number of observations:	6

Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} Conc (mg/l)	$10^{X95\%-}$ Conc (mg/l)	$10^{X95\%+}$ Conc (mg/l)
10	16.4	8.8	30.6
20	23.1	13.0	41.0
50	64.5	40.3	103.2
80	179.7	111.4	290.0

Regression line: Y= 67.38 X -71.91

Figure 1: Influence of [redacted] on the respiration rate of aerobic waste water (activated sludge) bacteria (values in % of the control).
Dashed curves represent the 95 % confidence limits.

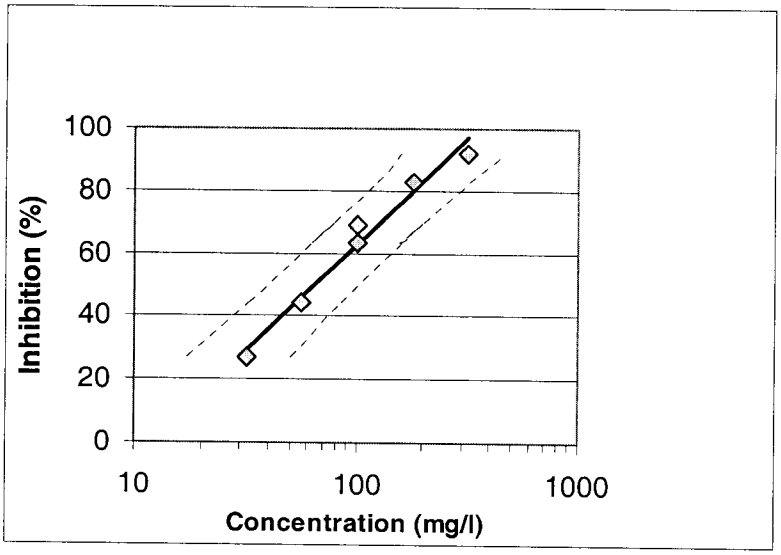


Table 3: Determination of the EC50-value for 3,5-dichlorophenol.

Parameter: % inhibition

Concentration (mg/l)	X Log conc. (mg/l)	Y Inhibition (%)
3.2	0.505	13
10	1.000	52
32	1.505	77

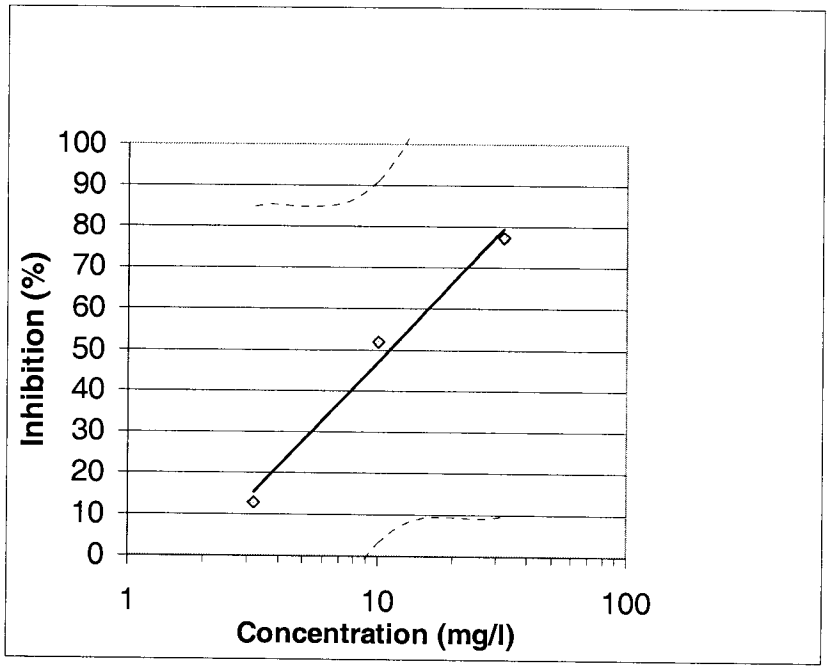
Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
50	11.1	0.5	263.0

Regression line: Y= 63.95 X -16.84

Slope:	63.9497
Intercept:	-16.8359
Multiple R:	0.9914
n = number of observations:	3

Figure 2: Influence of 3,5-dichlorophenol on the respiration rate of aerobic waste water (activated sludge) bacteria (values in % of the control).
Dashed curves represent the 95 % confidence limits.



[redacted]

[redacted]

Certificate of Analysis

[redacted]

ICS-331

Product name : [redacted]

Chemical name : [redacted]

Batch number : **VRS 01048**

Test results:

Method	Analysis of	Unit	Result *1
Col/86.2, Jo/95.2	Peroxidic compounds (sum) ***See page 2 for a specification***	% m/m	28.9 (± 1.5)
J20010381	[redacted]	% m/m	66.0 (± 1.0)
J20010381		% m/m	2.7 (± 0.3)
Amp/88.9	Water	% m/m	2.8 (± 0.3)
J20010381	Unidentified impurities	% m/m	0.5 (± 0.2)

*1 bracketed values are estimated 95% confidence intervals

File code : **TNA-2001004**

Analytical documentation : 20010381

[redacted]

[redacted]

[redacted]

Certificate of Analysis

[redacted]

[redacted] batch VRS 01048: specification of the peroxidic compounds

structure	% m/m
[redacted]	

[redacted]